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13. ABSTRACT *(Maximum 200 words)*

A research group was developed to pursue basic and clinical research in immunology with emphasis on AIDS. Appropriate space within the Guthrie Research Institute was renovated and equipped and investigators were recruited to occupy these facilities. The research laboratories are occupied by Drs. Henderson, Noti and Kestler, while a clinical research laboratory was also developed. The basic research laboratories are investigating the pathogenesis of AIDS with emphasis on viral entry/fusion receptor molecules expressed on human cells, the regulation of expression of human integrin genes, and expression of regions of the HIV envelope genes by recombinant means for functional studies. The clinical laboratory has made available numerous tests for leukocyte function, immunosuppression and and evaluation of HIV status. The basic research program has identified human cellular proteins that appear to play a direct role in HIV entry/fusion and is beginning to clone the genes which encode these proteins, has developed recombinant HIV envelope protein that contains cellular interaction domains and plays a role in pathogenesis of HIV disease and has identified important regulatory domains of the 5' sequences of the integrin gene p150/95.

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TITLE OF PROJECT: Development of a Medical Research Institute Directed at Basic and Clinical Research Immunology

PROJECT PERIOD: August 1, 1989 - July 31, 1992

GRANT NUMBER: DAMD17-89-Z-9042

PRINCIPAL INVESTIGATORS: Robert E. Hall, M.D., Ph.D. (8/1/89-10/1/91), Lee A. Henderson, Ph.D. (10/1/91 - 7/31/92)

(a) Front Cover

(b) Report Documentation (SF298)

(c) Foreword

(d) Table of Contents

(e) INTRODUCTION

The immune system plays a crucial role in defense against a host of human diseases, including bacterial and viral infections, cancer, rheumatologic diseases, and immune deficiency diseases, of which AIDS represents the most important new serious public health threat of the 20th century. A detailed understanding of the normal immune system, as well as the pathophysiology of the abnormal immune system, are therefore of great importance to the effective prevention and treatment of a variety of diseases (including AIDS) which might be encountered by military personnel. This project was designed to expand and further develop the Guthrie Research Institute, a medical research institute directed at basic and clinical research in immunology. Further, the project was designed to promote basic/clinical research collaborative studies of human immunologic diseases with the Guthrie Clinic, with which the Guthrie Research Institute is closely affiliated. Specific Aims of this project included (1) renovation of existing space to create three new basic immunology research laboratories and one Clinical Research Immunodiagnostic Laboratory; (2) recruit, equip, and provide start-up funding for the three new basic research laboratories, at least one of which will devote a majority effort toward AIDS; and (3) equip and provide start-up funding of the Clinical Research Immunodiagnostic Laboratory for the study of immunologic diseases, including HIV-infected individuals. In this Final Report, we will document how these goals were accomplished. We believe that completing these goals has contributed to building a strong basic scientific base in immunology for the Army, which should help in better understanding and treating AIDS and other diseases of importance to the military.

(f) BODY

Specific Aim1: Renovation of Existing Space to Create Three New Basic Immunology Research Laboratories and one Clinical Research Immunodiagnostic Laboratory (CRIL).

During the first project year, architectural plans were drawn up and bids requested to renovate approximately 8,000 square feet of space on the third floor of the Guthrie Research Institute building and approximately 400 square feet on the first floor (for the CRIL). Bids were requested according to DOD guidelines, the lowest bid accepted, and the renovation project completed. This included an approximately 1,000 square foot state-of-the-art BL3 containment facility for use in experiments involving HIV or other infected materials. Similarly, basic large equipment (centrifuges, gamma and beta counters, coldroom, spectrophotometer, etc.) was purchased after receiving at least three bids. The new facility was opened on August 1, 1990.

Specific Aim 2: Recruit, Equip, and Provide Start-up Funding For the Three New Basic Research Laboratories, At Least One of Which Will Devote a Majority Effort Toward AIDS.

In the 8,000 square feet of basic research space on the third floor of the Research Building, the BL-3, a common equipment room and three contiguous research laboratories were housed. Three new independent investigators were recruited to the Guthrie Research Institute. Each laboratory was adequately equipped and start-up supply funds provided for research supplies. All laboratories at the Guthrie Research Institute, including these three new laboratories, are currently engaged in submitting proposals for additional future funding of research to Non-Army sources of funding (including NIH and other non-governmental agencies). In addition, a renewal application to continue the research started during this project period has been submitted to the Army.

A brief description of each new laboratory is provided below; a more detailed summary of research activities during the project period is provided later in the BODY of this report. An important goal accomplished in this project is that these three laboratories collaborate significantly in the area of HIV immunobiology. An updated Curriculum Vitae for each new investigator is also provided in this report.

John D. Noti, Ph.D. was a Research Associate in Dr. Hall's laboratory (2nd floor, research space which antedated this grant) until 7/1/89 at which time he was promoted to Assistant Scientist (equivalent to Assistant Professor) and moved into one of the newly-renovated laboratories on the third floor. Dr. Noti's research interests include the molecular biology of leukocyte adhesion molecules, HIV-leukocyte receptors, and tumor cell adhesion molecules involved in generation of metastases.

Lee A. Henderson, Ph.D. was an established HIV immunologist on the faculty of Tulane University Medical School and joined the Guthrie Research Institute on December 1, 1990, as Director of the BL-3 Facility and independent research investigator. In view of his more senior status, Dr. Henderson actively advised the careers of Drs. Noti and Kestler (below), and was integral to developing collaborative efforts in HIV research among these three labs. Dr. Robert Hall was Scientific Director of the Guthrie Research Institute and Principal Investigator of this project until 10/1/92 at which point he moved his laboratory to the University of Tennessee.

Dr. Henderson was the logical choice to assume the role as Principal Investigator of this project, and this was proposed to and approved by the Army. Dr. Henderson has continued to actively investigate HIV virus-leukocyte receptors, including CD4 and non-CD4 mechanisms, as well as the mechanism of viral infection.

Daniel P. Kestler, Ph.D. was a Research Associate at Indiana University School of Medicine prior to joining the Guthrie Research Institute on 9/1/91 as independent investigator. Dr. Kestler is an immunologist and molecular biologist whose current research interests include HIV immunology, the molecular biology of HIV-leukocyte receptor interactions, and RNA and DNA tumor virus leukemagenesis.

Summary of Research Results:

GP41 Interaction with nonCD4 proteins on Human Cells.

A gp41 peptide, CS3, interacts with a polypeptide complex of 200-250 Kd on human cells that minimally consists of two polypeptides of 45 and 80 Kd (p45 and p80, respectively). The 45 Kd protein is not detected on murine cells. Both CS3 and carrier conjugates of CS3 (CS3-HSA) bind to p45 and p80. Furthermore, CS3-HSA (0.1-10 ug/ml peptide or 40 nM) blocks HIV infection to the extent of 75-90% depending on the isolate of HIV used. Rabbit polyclonal antibody to p80 recognizes both the 200-250 Kd complex which binds to CS3 and immunoprecipitates p80. Anti-p80 sera also blocks HIV infection with an IC_{50} at a dilution of 1:1000. CS3 interacts with human cells with high affinity binding to two distinct sites of 35,000 and 140,000 molecules per cell and dissociation constants of 10^{-8} and 10^{-7} M, respectively. Both p45 and p80 have been purified by affinity chromatography or conventional means including a final step on elution from a reverse phase (C4) column in the presence of an acteonitrile/isopropanol gradient. Preliminary studies with a recombinant form of gp41 show that it blocks CS3 binding with an affinity (pseudoaffinity) constant 100 fold greater than peptide itself.

The ability of CS3-HSA and anti-p80 to block HIV infection is either through direct interference with the ability of viral gp41 to interact with this putative receptor or by altering the activation state of the cell in a manner that renders cells refractory to HIV infection. Future plans are to clone the genes which encode p45 and p80, determine whether viral gp41 directly interacts with the p45/p80 complex and the development of peptide analogues for mimetics drug design and use in therapy for AIDS.

Revelant publications:

M. Nasar Qureshi, David H. Coy, Robert F. Garry and Lee A. Henderson. Characterization of a putative cellular receptor for HIV transmembrane glycoprotein using synthetic peptides. AIDS 4:553, 1990.

William R. Gallaher, Lee A. Henderson, Cesar Fermin, Ronald C. Montelero, Angela Martin, M. Nasar Qureshi, Judith M. Ball, Quentin Sattentau, Hong Luo-Zang, and Robert F. Garry. Membrane Interactions of HIV: Attachment, Fusion, and Cytopathology. In, Advances in Membrane Fluidity (Eds. R.C. Aloia and C.C. Curtain), 6:113-142, 1992.

Lee A. Henderson, Nasar Qureshi, Daniel Kestler, and John Noti. Novel gp41 binding proteins may play a direct role in HIV-1 infection: Anti-receptor antibody blocks HIV infection. Manuscript submitted.

Lee A. Henderson. Characterization of receptor complex that interacts with a peptide derived from HIV gp41 . Manuscript in preparation.

Daniel P. Kestler, Lee A. Henderson and John Noti. Construction and expression of recombinant HIV-1 gp41 constructs in prokaryotes. Manuscript submitted.

Transcriptional Regulation of the Leukocyte Integrins.

A key factor in understanding the mechanisms of cell-cell and cell-matrix interactions in the immune system has been the discovery of leukocyte-specific cell surface receptors involved in these interactions. These receptors, referred to as the leukocyte integrins, consist of three molecules, LFA-1, Mac-1, and p150-95. Elucidation of the molecular mechanisms that govern the expression of the leukocyte integrins will facilitate studies of adhesive interactions in the immune system. We have, therefore, begun studies designed to elucidate the transcriptional regulation of these molecules. The genomic sequences for the p150-95 and LFA-1 α subunits were isolated in this laboratory and we have extensively characterized the p150-95 sequence (appended manuscript). We have begun to identify regulatory elements that essential for this gene's expression in the pre-macrophage cell lines, U937 and HL60, and are expanding our efforts to include similar studies of the LFA-1 α subunit. Currently, using deletion mutagenesis and DNase I footprinting analysis, we have identified both silencer and enhancer elements that regulate p150-95 gene expression. Efforts to identify the binding proteins that interact with these elements are underway.

Isolation of Genes Associated with Breast Tumor Metastasis.

The major cause of death in most malignant diseases is not the primary tumor, but rather the metastatic spread of tumor cells to distant sites within the body. Metastasis is a complex multistep process that is undoubtably regulated by many different genes including the integrin genes. We are using the subtractive hybridization technique to select for genes that are differentially expressed in normal and metastatic breast cells. As sources of metastatic and non-metastatic cells we are using established breast cell lines and surgically-isolated tissue.

Relevant publications.

Noti, J.D., Gordon, M., and Hall, R.E. (1992). Human p150-95 α subunit: Genomic organization and analysis of the 5' flanking region. DNA and Cell Biol. 11, 123-138.

Noti, J.D. and Gordon, M. Intron 1 of the human p150-95 α subunit gene contains enhancer and PMA-responsive elements essential for transcription. Manuscript in preparation.

Specific Aim 3: Equip and Provide Start-up funding of the Clinical Research Immunodiagnostic Laboratory for the Study of Immunologic Diseases, Including HIV-infected Individuals.

As described in Specific Aim 1, approximately 400 square feet of space on the first floor of the research building was renovated to house a new Clinical Research Immunodiagnostic Laboratory (CRIL). The goal of this facility was to perform detailed immunologic evaluations of patients with immunologic diseases. Such diseases were intended to include both hereditary and acquired immunologic diseases. Such diseases were intended to include both hereditary and acquired immunodeficiency diseases (HIV and non-HIV immunodeficiencies) as well as allergic, rheumatologic, and malignant diseases. Although a stated goal of this project was to provide new information relevant to AIDS and HIV infection, our stated rationale was that information relevant to HIV could be gained by studying other human immunodeficiency states. The CRIL was also intended to provide "spin-off" ideas which might generate important new basic research projects relevant to human immunologic disease states. Dr. Hall served as Supervisor of the CRIL until 10/91, at which time Dr. Henderson became CRIL Supervisor and Principal Investigator of this project (see above).

In collaboration between Dr. Hall (Scientific Director and Principal Investigator until 10/91), other Guthrie Scientists, and Dr. Joseph Follett (Chief, Section of Allergy and Clinical Immunology at the Guthrie Clinic) the following laboratory evaluations were set up and were made freely available to Guthrie Clinic physicians for immunologic evaluation of patients. These tests were considered a clinical research tool and not billed to the patient or insurance company.

CLINICAL RESEARCH/IMMUNODIAGNOSTIC LABORATORY (CRIL)
Laboratory Evaluations, June 1, 1990

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1. Flow Cytometry
   a. Surface Antigen Phenotype of Acute and Chronic Leukemias (Peripheral Blood and Bone Marrow)

   b. T and B Cell Subsets (Non-Malignant) For Immunodeficiency Workup and Immune Evaluation

   c. Cell cycle/Ploidy Analysis of Select Neoplasms (e.g., Endoscopic Biopsies of Colon)

2. Lymphocyte Transformation/ Proliferation

   a. To Mitogens (Phytohemagglutinin, Con A, Pokeweek Mitogen)

   b. To Antigens (PPD, Candida, Mumps)

   c. To Drugs (Evaluation of Drug Hypersensitivity)

3. Neutrophil Function Studies (Immunodeficiency Workup)

   a. Nitroblue Tetrazolium (NBY) Reduction

   b. Chemotaxis

   c. Chemiluminescence

4. Cytotoxicity Assays
   a. Cytotoxic T Lymphocytes (Cell Mediated Lympholysis)

   b. Natural Killer Cell Assay To Tumor Cells

5. Select Complement Components (e.g., C1 inhibitor)

6. Allergy Studies

   a. Total Serum IgE (Radioimmunoassay)

   b. Allergen-Specific IgE By RAST (Radioallergosorbent Test)

7. Lymphocytotoxic Antibody Assay

With recruitment of Dr. Henderson and other HIV scientists, the following HIV in vitro evaluation were added to the available list:

1. Western blot analysis for HIV antibody specificity.
2. Viral titres in sera and titre produced by PBMC following mitogen activation.
3. Soluble CD4 and AZT sensitivity of clinical viral isolates.

(g) CONCLUSIONS

In conclusion, all of the goals of the original project proposal have been accomplished within the project period. Although this project was intended to be largely developmental, nevertheless modest basic research progress has been accomplished in this short time frame (considering that new investigators were not "on board" at the start of the project, but were phased in over the three years). More importantly, careers of promising young investigators have been launched, and this is expected to create important scientific dividends for many years to come. The Guthrie Research Institute has maintained a sincere desire to collaborate directly with Army Scientists in order to directly assist in Army research projects, and it is hoped that these collaborations as well as with other HIV research laboratories will develop in the future. In addition, present and future research at the Guthrie Research Institute should add to the scientific base available to Army scientists in order to better diagnose and treat AIDS and other diseases in which the immune system plays an important role. Finally, creation of the CRIL should facilitate communication between Guthrie Research Institute laboratories and the Guthrie Clinic, which will facilitate identification of new insights into AIDS and other immunologic disease as well as help communicate basic scientific finding to clinicians.

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Human p150,95 α-Subunit: Genomic Organization and Analysis of the 5′-Flanking Region

JOHN D. NOTI,* MARIELLA GORDON,* and ROBERT E. HALL†,‡

ABSTRACT

LFA-1, Mac-1, and p150,95 comprise a family of cell-surface glycoproteins that mediate adhesive interactions of myeloid and lymphoid cells. These glycoproteins are heterodimers composed of a common β-subunit and distinct α-subunits. The chromosomal gene for the α-subunit of p150,95 was isolated to provide a framework from which to study the mechanisms for expression of the gene. The gene spans 30 kb of DNA and contains 31 exons. In agreement with a previous report by Corbi *et al.* (1990), the exons were found to be divided into five groups separated by large introns. The extracellular domains are encoded in exons 2 through 30 while the transmembrane and cytoplasmic domains are encoded in exons 30 and 31. We have expanded these findings in a number of ways. The first exon contains the 5′ untranslated region. The 2,163-bp 5′-flanking sequence contains the first intron and several putative transcriptional initiation sites preceded by two TATA sequences and two GC-like boxes. Additional sequence motifs for a variety of DNA-binding proteins are present and discussed. Fusions of the bacterial chloramphenicol acetyltransferase gene (CAT) to approximately 5.3 kb of 5′-flanking DNA and also to subcloned fragments of this region were constructed and transfected into the human promonocytic cell line, U937. CAT expression was inducible with phorbol-12-myristrate-13-acetate (PMA) and full expression was dependent on the presence of intron 1 and sequences upstream from the 2,163-bp flanking DNA. Additionally, intron 1 and a region further upstream contain functional *cis*-acting sequences.

INTRODUCTION

THE INTEGRIN FAMILY of cell-surface receptors is extensively involved in cell-cell and cell-matrix interactions of the immune system. These receptors are glycoproteins that are composed of noncovalently associated α- and β-subunits (Hynes, 1987; Williams and Barclay, 1988; Kishimoto *et al.*, 1989; Tedder *et al.*, 1989; Larson and Springer, 1990). The integrin family is divided into three subfamilies that have unique β-subunits but distinct α-subunits. The β_2 subfamily contains three receptors, LFA-1, Mac-1, and p150,95 which are present on leukocytes and mediate a broad spectrum of adhesive-dependent functions. A heritable deficiency, leukocyte adhesion deficiency (LAD) of all three receptors from the leukocytes of affected individuals is characterized by severe, recurrent bacterial infections that are life-threatening (Fischer *et al.*, 1988). The genetic deficiency is due to mutations in the common β-subunit.

In addition to the involvement of these receptors in inflammatory phenomena, these molecules also are involved in the killing of tumor cells by cytotoxic T cells (CTLs) (Davignon *et al.*, 1981), natural killer (NK) cells, and lymphokine-activated killer cells (LAK) (Schmidt *et al.*, 1985); Martz, 1987). Recent evidence also suggests a role for these receptors in immunosurveillance. Some lymphomas including the majority of Burkitt's lymphomas do not express LFA-1 on their cell surface (Clayberger *et al.*, 1987; Rousset *et al.*, 1989; Stauder *et al.*, 1989). These observations imply that a decreased expression of one or more adhesion molecules may be a means by which some tumors escape immunosurveillance.

The distribution of these molecules differs with the particular cell type. LFA-1 (Kurzinger *et al.*, 1981) is found on virtually all leukocytes whereas Mac-1 and p150,95 are found primarily on monocytes, macrophages, and granulocytes (Springer *et al.*, 1979, 1986). The first appearance of the leukocyte integrins occurs as stem cells begin to dif-

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ferentiate. LFA-1 is expressed on pre-B and late myeloblasts, whereas, Mac-1 and p150,95 are detected as monocyte and granulocyte differentiation increases and is consistent with the detection of increased expression of the β-subunit when HL-60 promyelocytic leukemia cells differentiate *in vitro* along the granulocytic pathway (Miller *et al.*, 1986). LFA-1 and p150,95 are also found on CTLs and NK cells, although higher amounts of LFA-1 are expressed on both cell types (Lanier *et al.*, 1985). p150,95 is also highly expressed on hairy leukemia cells and, as such, has been used as an immunodiagnostic marker for this disease (Schwarting *et al.*, 1985). Recently, this receptor has been found on some B-cell chronic lymphocytic leukemias, some large-cell lymphomas of uncertain origin, and also on some neoplastic T-cell (Chadburn *et al.*, 1990).

The amino acid sequences of all three α-subunits and the common β-subunit have previously been determined (Corbi *et al.*, 1987; Law *et al.*, 1987; Arnout *et al.*, 1988; Larson *et al.*, 1989). To understand the molecular basis for the expression and function of the leukocyte integrins, we (Noti *et al.*, 1990) and others (Corbi *et al.*, 1990) have begun to isolate the genomic sequences for these molecules and preliminarily characterize these genes. In these findings we report the isolation of a cosmid clone that contains the complete transcriptional unit of the p150,95 α-subunit gene. We have determined the exon-intron organization of this gene and have localized the transcriptional start site(s). DNA sequence and functional analysis were done to identify transcriptionally important regions in the 5'-flanking DNA. It is anticipated that this work will facilitate studies concerning the regulation of the p150,95 α-subunit at the transcriptional level.

MATERIALS AND METHODS

Construction and screening of the genomic library

A human genomic library was constructed in the cosmid pWE15 (Wahl *et al.*, 1987) from DNA isolated from human peripheral blood leukocytes. A total of 2×10^6 recombinant clones were screened by colony hybridization with a partial cDNA clone for the p150,95 α-subunit isolated from an HL60 cDNA library.

DNA sequence analysis

Double-stranded DNA was sequenced by the dideoxy chain termination method with the T, DNA polymerase sequenase according to the manufacturer's (U.S. Biochemicals, Cleveland, OH) instructions. DNA fragments were subcloned into the plasmid pBlueskript SK⁺ (Short *et al.*, 1988) prior to sequencing. Cosmid DNA (10 μg) was also sequenced directly.

S1 nuclease and primer extension analysis

A modified method of Berk and Sharp (Berk and Sharp, 1977) was used for S1 nuclease analysis. Single-stranded probes were prepared from appropriate DNA fragments subcloned into the plasmid m13mp18. Poly(A)⁺RNA (10–25 μg) isolated from the human promonocytic cell line U937 was annealed to the probe in 20 μl of 80% formamide/0.4 *M* NaCl/50 m*M* PIPES pH 6.4/1 m*M* EDTA for 18 hr at either 45°C or 50°C. Then, 300 μl of an S1 cocktail buffer (250 m*M* NaCl/50 m*M* NaOAc pH 4.5/4.5 m*M* $ZnSO_4$/5 μg salmon sperm DNA/400 units S1 nuclease) was added for 60 min at 37°C. The reaction products were analyzed on a polyacrylamide sequencing gel.

For primer extension analysis, 10 μg of poly(A)⁺RNA was annealed with 3–4 ng of the appropriate end-labeled oligonucleotide. Annealing was done in 10 μl of 0.4 *M* NaCl/40 m*M* PIPES pH 6.4/5 m*M* EDTA for 16 hr at 30°C. Ninety microliters of a cocktail solution (30 m*M* Tris-HCl pH 8.3/10 m*M* $MgCl_2$/10 m*M* dithiothreitol/0.5 m*M* each dNTP/60 units placental RNase inhibitor/60 of ng actinomycin D/55 units AMV reverse transcriptase) was then added followed by incubation for 90 min at 42°C. The products were analyzed on a polyacrylamide sequencing gel.

Construction of CAT plasmids

The plasmid pB4P3-CAT was constructed by first subcloning the 746-bp *Bam* HI–*Pst* I genomic fragment (corresponding to the region −1,020 to −1,766 from Fig. 2) from clone 8B into the polylinker of pBluescript SK⁺. The resulting plasmid was then digested with *Hin*d III and *Xba* I (restriction sites that flank the *Bam* HI–*Pst* I fragment) and the fragment containing the 746-bp *Bam* HI–*Pst* I fragment was then isolated and ligated into the *Hin*d III–*Xba* I sites upstream from the CAT gene in the promoterless CAT reporter plasmid, pCAT-Basic (Promega, Madison, WI). The plasmid pB4.1-CAT was constructed by first subcloning the 4.4-kb *Bam* HI genomic fragment (see Fig. 2) from clone 8B into pBluescript SK⁺. The resulting plasmid was then digested with *Hin*d III and *Xba* I and the fragment containing the 4.4-kb *Bam* HI fragment was then ligated into *Hin*d III–*Xba* I-digested pCAT-Basic. The plasmid pB4,3A-CAT was constructed by first subcloning a 1.1-kb *Bam* HI–*Pst* I genomic fragment containing intron 1 and the ATG start codon for the p150,95 α-subunit from clone 8B into pBluescript SK⁺. The resulting plasmid was then digested with *Xba* I (located 12 bp from the *Bam* HI site) and *Sac* I (located 32-bp upstream from the ATG codon for the p150,95 α-subunit gene, Fig. 2). The *Sac* I terminus of this fragment was ligated to a *Sac* I–*Xba* I adaptor prepared by annealing the two oligonucleotides, 5'-GTCGACGTAGCCGTTTGGTACCCCTGATAG-3' and 5'-CTAGCTATCAGGGGTACCAAACGGCTACGT CGACAGCT-3'. Four translational stop codons (underlined) in two different reading frames were introduced into this adaptor. The resulting 1,038-bp *Xba* I fragment (containing intron 1 but lacking the ATG codon) was ligated into the *Spe* I site located 49 bp upstream of the the ATG codon for the CAT gene in pB4.1-CAT. The plasmid pB4,3B-CAT was constructed by ligating the intron 1-containing *Xba* I fragment in the opposite orientation into the *Spe* I site of pB4.1-CAT. DNA sequence analysis confirmed that the various fragments were ligated correctly into each parent plasmid. The plasmids pSVCat-1 and

pSVCAT-2 were constructed by ligating the 1.1-kb *Bam* HI-*Pst* I fragment containing intron 1 and the 4.4-kb *Bam* HI fragment from clone 8B, respectively, into the enhancerless SV40 promoter plasmid, pCAT-Promoter (Promega, Madison, WI). The plasmid CMV-CAT was constructed by ligating the 0.7-kb *Sau* 3A fragment containing the CAT gene from the pCAT-Basic plasmid into the *Bam* HI site of pcDNAI (Invitrogen Corp., San Diego, CA). The plasmid CMV-CAT contains the cytomegalovirus promoter/enhancer DNA fused to the CAT gene.

Cell culture

The cell line U937 was grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin-streptomycin.

Transfections and CAT assays

HeLa cells were transfected by the DEAE-dextran method (Gorman *et al.*, 1982) with modifications. HeLa cells (2×10^6) were transfected with 30 μg of plasmid DNA in 4 ml of DMEM supplemented with 10% NuSerum (Collaborative Research, Waltham, MA) and 100 μg of DEAE-dextran. After 4 hr at 37°C, the medium was then removed and 4 ml of DMEM supplemented with 10% NuSerum and 10% DMSO was added for 2 min at room temperature. The cells were washed and 15 ml of tissue culture medium was then added. PMA was added at (2 ng/ml) 24 hr later. The cells were harvested 72 hr after transfection.

Electroporation was used to transfect U937 cells. The cells were washed once with warm PBS and 2×10^7 cells were resuspended in 0.2 ml of RPMI-1640 supplemented with 10 m*M* dextrose, 0.1 m*M* dithiothreitol (DTT). After adding 50 μg of plasmid DNA in 20 μl of RPMI-1640/dextrose/DTT, the cells were then subjected to a single electric pulse (capacitance, 960 μF; voltage, 200 V; duration, 28–30 msec) in a BioRad gene pulsar apparatus. The cells were transferred to a 100-mm tissue culture dish containing 15 ml of tissue culture medium. PMA (2 ng/ml) was added 24 hr later. The cells were harvested 72 hr after electroporation.

The cells were harvested, resuspended in 100 μl of 0.25 *M* Tris-HCl pH 8.0 and lysed by freezing and thawing. After normalization for protein content, CAT activity was determined with a commercially available kit (Promega, Madison, WI). The reaction products were separated by thin-layer silica gel chromatography. The plates were autoradiographed, and the acetylated products were cut from the plate and counted in a scintillation counter. The CAT assays were performed in duplicate and repeated three to five times to ensure reproducibility.

Polymerase chain reaction analysis

The polymerase chain reaction (PCR) was done directly on poly(A)⁺RNA isolated from U937 cells transfected with pB4,3A CAT or pB4,3B CAT as described (Brenner *et al.*, 1989). First-strand cDNA was synthesizsed from 50–100 ng of poly(A)⁺RNA in a 20-μl volume with oligo(dT). Five microliters of the first-strand cDNA was amplified by PCR for 35 cycles with sense primers derived from the −1,627 to −1,059 region of the p150,95 γ-subunit gene (see Fig. 2) in combination with an antisense primer to the 5′ end of the CAT gene. The PCR products were analyzed on a 4% NuSieve agarose gel (FMC Corp., Rockland, ME).

RESULTS

Isolation of the genomic sequences for the p150,95 α-subunit

Five cosmid clones were isolated and analyzed by comparative restriction enzyme analysis and by hybridization with oligonucleotides specific for the amino-terminal, carboxy-terminal, and central portions of the p150,95 cDNA sequence. One cosmid clone, designated 8B, contained a 35-kb insert that hybridized with these oligonucleotide probes. Total genomic DNA digested with several restrictions enzymes and hybridized with clone 8B DNA as probe revealed a restriction enzyme pattern identical to the pattern of DNA fragments of the inserted DNA in this clone (data not shown). This indicated that the genomic DNA in clone 8B was from a contiguous region of chromosomal DNA. The restriction enzyme map of the region is shown (Fig. 1).

Exon–intron organization

The exon-intron organization was determined by DNA sequence analysis. An exon-intron boundary was indicated when the sequence from the genomic clone diverged from that of the cDNA (Corbi *et al.*, 1987). With this strategy more than 15 kb of DNA was sequenced. The DNA sequences of all the exon-intron junctions contained the invariant gt and ag intronic dinucleotides (Table 1). With the exceptions of exons 1 and 31 (detailed below), the size of each exon was less than 210 nucleotides. The exons encoding the protein sequence for the α-subunit of p150,95 (exons 2–31) span approximately, 30 kb of DNA (Fig. 1) and are clustered in five groups that correlate with previously identified domains of this protein (Corbi *et al.*, 1987; see Discussion).

Identification of an intron in the 5′-flanking region

The 2,163-nucleotide sequence upstream of the ATG translational start site was determined and shown in Fig. 2. Two putative 3′ splice acceptor consensus sequences are present 52 nucleotides and 65 nucleotides upstream from the ATG codon. To determine whether these are functional splice sites, S1 nuclease and primer extension analysis was performed. For S1 analysis, a 177-nucleotide single-stranded probe was prepared that spanned these two potential splice sites. This probe was then hybridized with poly(A)⁺RNA from PMA-stimulated U937 cells followed by S1 nuclease digestion. Two major and one minor protected fragments were detected. The size of these protected

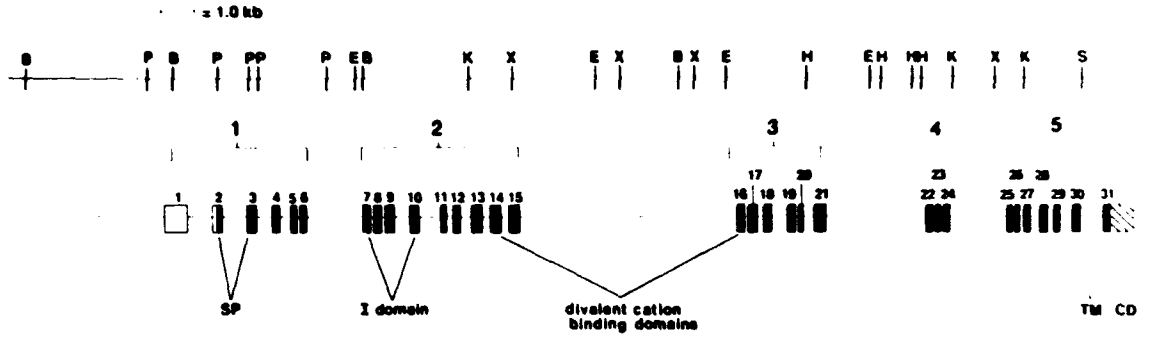


FIG. 1. The intron–exon organization of the p150,95 α-subunit gene. The exons are in solid black boxes and are numbered. The 5′ untranslated region is shown in open boxes and is separated by intron 1. The 3′ untranslated region is shown as a hatched box. A partial restriction enzyme map is shown: E, *Eco* RI; K, *Kpn* I; H, *Hind* III; S, *Sal* I; B, *Bam* HI; P, *Pst* I; X, *Xba* I. The exons are clustered into five groups as indicated. SP, Signal peptide; TM, transmembrane domain; CD, cytoplasmic domain.

fragments (Fig. 3) correlated with the position of the 3′ consensus splice sequences, thus, indicating the presence of an intron in the 5′-flanking region. This was confirmed by primer extension analysis with the N-6 oligonucleotide (Fig. 3). Extension of the N-6 oligonucleotide on poly(A)⁺RNA from PMA-stimulated U937 cells revealed several extension products that were considerably longer than the S1 nuclease protected fragments. The longest primer-extension product, 360 nucleotides, indicated that the mature mRNA for the p150,95 α-subunit extends at least this far upstream of the ATG translational start codon.

The 5′-flanking sequence was scanned for the presence of the 5′ splice donor site. A consensus splice sequence is present 650 nucleotides upstream of the ATG start codon. To determine if this is a functional splice site, primer extension analysis was done with primers tha flanked this site. As shown in Fig. 4, no distinct extension products were seen when oligonucleotides N-34 and N-35 (located 3′ of this site) were used as primers. However, extension products 254 and 301 nucleotides long resulted when oligonucleotide N-8 (located 5′ of this site) was used as the primer. This indicated that the consensus 5′ splice sequence located 650 nucleotides upstream of the ATG start codon is functional.

Determination of the site of transcriptional initiation

Initial primer extension analysis with the N-6 oligonucleotide resulted in two major extension products 270 and 360 nucleotides long (Fig. 3). Since the potential for generating prematurely terminated extension products increases with the length of the mRNA being transcribed, analysis was performed with primers that hybridized with sequences upstream of intron 1. The length of each primer extension product was obtained by comparison with the sequencing ladders in the adjacent lanes. Two extension products, 214 and 408 nucleotides long (corresponding to −1,178 and −1,371, respectively), were detected when primer N-9, the primer closest to the 5′ splice acceptor site of intron 1, was extended (Fig. 5). Primer N-24, which is located 60 nucleotide 5′ to primer N-9, was extended to 55 nucleotides in length (corresponding to −1,080) (Fig. 5). The heterogeneity of the sizes of the extended products may result from multiple transcriptional starts, or might be the result of an inability of reverse transcriptase to extend products beyond a secondary mRNA structure. The smallest extension products of the N-9 and N-24 primer are preceded by a 29-nucleotide A+T-rich sequence (−1,108 to −1,080) that potentially can form a stem-loop structure (Fig. 2). Conceivably, however, this A+T-rich sequence may serve as an alternative initiation site (Broders *et al.*, 1990). The results of extension reactions beginning 5′ to this A+T-rich region are shown in Fig. 5. Two major extension products, 81 and 189 nucleotides in length (corresponding to −1,200 and −1,371, respectively), were detected when N-22 was used as the primer.

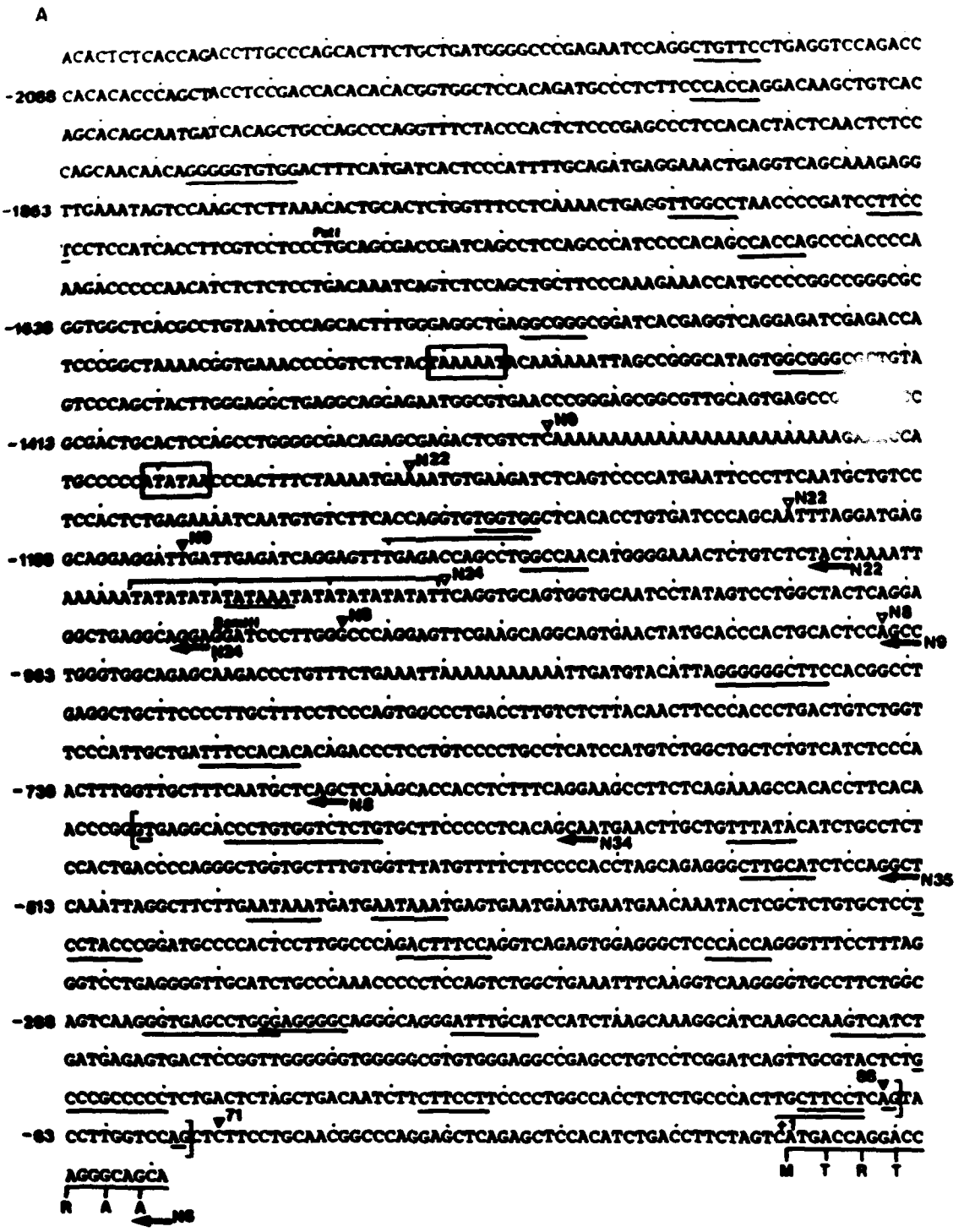
The upstream start sites at −1,308 and −1,200 are preceded by a TATA sequence (Matsui *et al.*, 1980) at −1,331. The longest extension product at −1,371 is upstream of this TATA sequence but is also preceded by a TATA-like sequence begining at −1,531 (TAAAAAT). Two sequences that share homology with the Sp1 consensus sequence are found within the region of the TATA sequences (Dynan and Tjian, 1983) (Fig. 2).

Analysis of the 5′-flanking sequence

The 5′-flanking sequence was analyzed (Ghosh, 1990) for other sequences that could potentially bind transcriptional regulatory proteins (Table 2). The sequence, GAACAG, at position −2,108 is homologous to a sequence motif in the glucocorticoid response element (GRE) in the Moloney murine leukemia virus (Mσ-MuLV) enhancer (speck and Baltimore, 1987). The sequence, CCACCA, present in the region upstream of the immunoglobulin heavy-chain enhancer (Augereau and Chambon, 1986) is also found upstream (positions −2,033 and −1,729) of the putative TATA boxes. At position −1,925 is a sequence identical to the GTI motif in the SV-40 enhancer (Siao *et al.*, 1987). The sequence GGCCAA is found at position −1,810 and is homologous to a low-affinity CTF/nuclear factor I binding site (Garcia *et al.*,

TABLE 1.

Exon no.	Exon size (bp)	Intron size (bp)	Sequence at exon-intron junction	
			5′ Splice donor	3′ Splice acceptor
1	714/777	594/607	CGG gtgaggcaccc	.....gcttcctcag TAC
2	102	500 ±50	ACA G gtgagcctgg	.....tctttcccag CC TTA
3	106	650 ±50	TCC TG gtgaggccca	.....ctcctcgcag G GTG
4	104	300 ±40	CAG G gtgagtcacc	.....tgtcccacag TG CCC
5	71	161	CTG gtgagttgcc	.....gtgtccccag GCC
6	112	2000 ±200	CAG G gtgagtgtct	.....ccttccccag AG TGC
7	131	85	CAG gtgtgctttg	.....cttctcccag TTT
8	146	200 ±50	GTC GT gtccgtcctg	.....ttcctgatgg G CAC
9	154	500 ±200	GGG gtaggcctgg	.....ctggggacag GTT
10	151	500 ±200	GAG G gtgagtctga	.....cttacccaag GT ACG
11	74	149	CCT gtgcgtgggg	.....ctttctccag GAT
12	130	300 ±150	CTG G gtgagaaaca	.....ggcccctcag GT TAC
13	143	180 ±20	CAG gttgggcgtg	.....tg.. ttcag ATC
14	141	88	GGG gtgagttgct	.....ttttctgcag TGG
15	210	6500 ±200	CAG gtgaggccgt	.....ctctggccag CGG
16	131	131	CTC AG gtgagagcag	.....ctgcccgcag GA CCA
17	163	100 ±40	AGC C gtgagtcccc	.....ccgcctacag GT GAC
18	155	500 ±100	CCG gtgcgtctgg	.....cgtcccccag AGC
19	132	100 ±30	TCC gtgagtcctg	.....tcccacgcag CTA
20	74	500 ±150	CCA GG gtgagcgccc	.....tgatacccag CT TGA
21	142	3500 ±200	CAG gtgcaccctc	.....tcatgcctag AAA
22	117	84	CAG gtcagcctgg	.....cctttcctag ATC
23	80	99	AGC AG gtgagccggg	.....ctttctccag T GAG
24	84	2100 ±100	AGC AG gtcagcaggt	.....ccctttccag C CAC
25	79	108	CAG gtcaggtggtg	.....ctctgtgcag GTC
26	108	76	CAG gtacctggat	.....tttcccccag AAC
27	84	200 ±50	CTG gtgaggaggg	.....ctgcccccag GAC
28	114	73	CAG gtgtgtgggt	.....ccctttgcag ATA
29	102	250 ±40	CAG gtagagacca	.....tccacttcag ACG
30	111	1000 ±100	AAA gtgagtgttt	.....ttcccccctag GTT
31	1247			


A
ACACTCTCACCAGACCTTGCCCAGCACTTCTGCTGATGGGGCCCGAGAATCCAGGCTGTTCCTGAGGTCCAGACC
-2068 CACACACCCAGCTACCTCCGACCACACACACGGTGGCTCCACAGATGCCCTCTTCCCACCAGGACAAGCTGTCAC
AGCACAGCAATGATCACAGCTGCCAGCCCAGGTTTCTACCCACTCTCCCGAGCCCTCCACACTACTCAACTCTCC
CAGCAACAACAGGGGGTGTGGACTTTCATGATCACTCCCATTTTGCAGATGAGGAAACTGAGGTCAGCAAAGAGG
-1863 TTGAAATAGTCCAAGCTCTTAAACACTGCACTCTGGTTTCCTCAAAACTGAGGTTGGCCTAACCCCGATCCTTCC
TCCTCCATCACCTTCGTCCTCCCTGCAGCGACCGATCAGCCTCCAGCCCATCCCCACAGCCACCAGCCCACCCCA
AAGACCCCCAACATCTCTCTCCTGACAAATCAGTCTCCAGCTGCTTCCCAAAGAAACCATGCCCCGGCCGGGCGC
-1438 GGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCTGAGGCGGGCGGATCACGAGGTCAGGAGATCGAGACCA
TCCCGGCTAAAACGGTGAAACCCCGTCTCTACTAAAAATACAAAAAATTAGCCGGGCATAGTGGCGGGCGCCTGTA
GTCCCAGCTACTTGGGAGGCTGAGGCAGGAGAATGGCGTGAACCCGGGAGCGGCGTTGCAGTGAGCCG
-1413 GCGACTGCACTCCAGCCTGGGGCGACAGAGCGAGACTCGTCTCAAAAAAAAAAAAAAAAAAAAAAAGA
TGCCCCCTATATACCCACTTTCTAAAATGAAAATGTGAAGATCTCAGTCCCCATGAATTCCCTTCAATGCTGTCC
TCCACTCTGAGAAAATCAATGTGTCTTCACCAGGTGTGGTGGCTCACACCTGTGATCCCAGCAATTTAGGATGAG
-1188 GCAGGAGGATTGATTGAGATCAGGAGTTTGAGACCAGCCTGGCCAACATGGGGAAACTCTGTCTCTACTAAAATT
AAAAAATATATATATATAAATATATATATATATTCAGGTGCAGTGGTGCAATCCTATAGTCCTGGCTACTCAGGA
GGCTGAGGCAGGAGGATCCCTTGGGCCCAGGAGTTCGAAGCAGGCAGTGAACTATGCACCCACTGCACTCCAGCC
-963 TGGGTGGCAGAGCAAGACCCTGTTTCTGAAATTAAAAAAAAAAATTGATGTACATTAGGGGGGCTTCCACGGCCT
GAGGCTGCTTCCCCTTGCTTTCCTCCCAGTGGCCCTGACCTTGTCTCTTACAACTTCCCACCCTGACTGTCTGGT
TCCCATTGCTGATTTCCACACACAGACCCTCCTGTCCCCTGCCTCATCCATGTCTGGCTGCTCTGTCATCTCCCA
-738 ACTTTGGTTGCTTTCAATGCTCAGCTCAAGCACCACCTCTTTCAGGAAGCCTTCTCAGAAAGCCACACCTTCACA
ACCCGGCTGAGGCACCCTGTGGTCTCTGTGCTTCCCCCTCACAGCAATGAACTTGCTGTTTATACATCTGCCTCT
CCACTGACCCCAGGGCTGGTGCTTTGTGGTTTATGTTTTCTTCCCCACCTAGCAGAGGGCTTGCATCTCCAGGCT
-513 CAAATTAGGCTTCTTGAATAAATGATGAATAAATGAGTGAATGAATGAATGAACAAATACTCGCTCTGTGCTCCT
CCTACCCGGATGCCCCACTCCTTGGCCCAGACTTTCCAGGTCAGAGTGGAGGGCTCCCACCAGGGTTTCCTTTAG
GGTCCTGAGGGGTTGCATCTGCCCAAACCCCCTCCAGTCTGGCTGAAATTTCAAGGTCAAGGGGTGCCTTCTGGC
-288 AGTCAAGGGTGAGCCTGGGAGGGGCAGGGCAGGGATTTGCATCCATCTAAGCAAAGGCATCAAGCCAAGTCATCT
GATGAGAGTGACTCCGGTTGGGGGGTGGGGGCGTGTGGGAGGCCGAGCCTGTCCTCGGATCAGTTGCGTACTCTG
CCCGCCCCCTCTGACTCTAGCTGACAATCTTCTTCCTTCCCCTGGCCACCTCTCTGCCCACTTGCTTCCTCAGTA
-63 CCTTGGTCCAGCTCTTCCTGCAACGGCCCAGGAGCTCAGAGCTCCACATCTGACCTTCTAGTCATGACCAGGACC
AGGGCAGCA
M T R T
R A A
B
P TAAAAAT ATATAA B
-1766 -1531 -1371 -1331 -1308 -1200 -1025 +1
intron 1 ATG

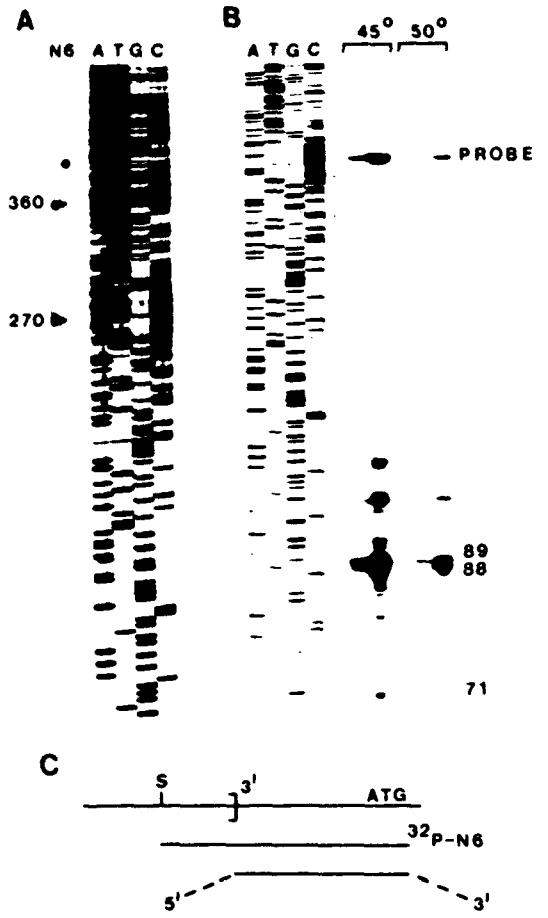


FIG. 3. Location of intron 1 in the 5′ untranslated region. A. Primer extension analysis of U937 poly(A)⁺-RNA with the N-6 primer is shown. The size of the longest extension product (360 nucleotides) is shown next to a DNA sequencing ladder. B. S1 nuclease analysis of U937 poly(A)⁺RNA is shown. Hybridizations were done at either 45°C of 50°C prior to the addition of S1 nuclease. The samples (1 μl and 5 μl) were loaded adjacent to a DNA sequence ladder obtained with the N-6 primer. The major protected products (88 and 89 nucleotides) and a minor protected product (71 nucleotides) are indicated. Undigested probe DNA is shown. C. A schematic of the S1 nuclease reaction is shown. ^{32}P-Labeled N-6 primer was extended to the *Sau* 3A (S) site with Klenow enzyme to generate a 177-nucleotide single-stranded probe. The 3′ site of intron 1 is shown. U937 Poly(A)⁺RNA that is not homologous to the probe is indicated with a dotted line.

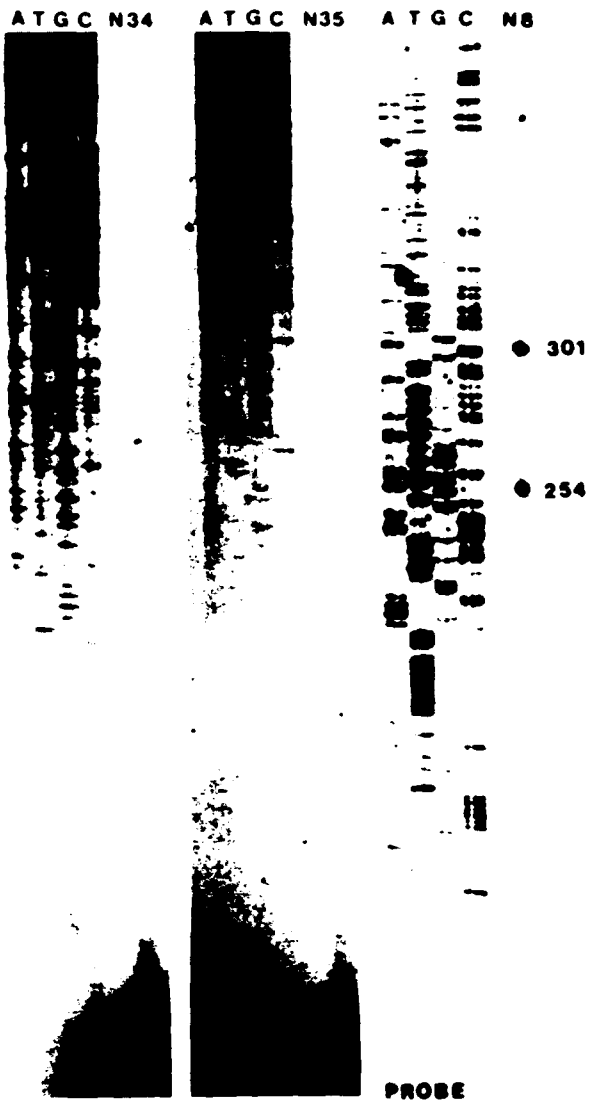


FIG. 4. Location of the 5′ end of intron 1. Primer extension analysis of U937 poly(A)⁺RNA with the N-8, N-34, and N-35 primers is shown. The N-34 and N-35 primer-extension products (1-μl and 5-μl samples) were loaded adjacent to a DNA sequence ladder. The N-8 primer extension products are shown next to a DNA sequence ladder. The size of each extension product in nucleotides is indicated.

FIG. 2. Nucleotide sequence of the 5′-flanking region. A. The 2,163-nucleotide sequence upstream from the ATG translational start site designated +1 is shown. The 5′ and 3′ consensus splice sites are in brackets. The location of the splice sites determined by S1 nuclease analysis are indicated with closed arrowheads. The numbers 88 and 71 adjacent to the closed arrowheads refer to the size of the major and the minor protected fragments (see Fig. 4). The location of the oligonucleotides used for primer extension and S1 nuclease analysis are indicated with horizontal arrows. The transcriptional start sites are indicated with open arrowheads and the primer used to obtain that particular site is shown next to the arrow. Putative TATA sequences are boxed; the potential binding sequences for transcriptional factors are underlined; and a potential hairpin loop is overlined. B. A schematic of the transcriptional start sites in relation to the putative TATA boxes is shown. The numbering corresponds to the DNA sequence.

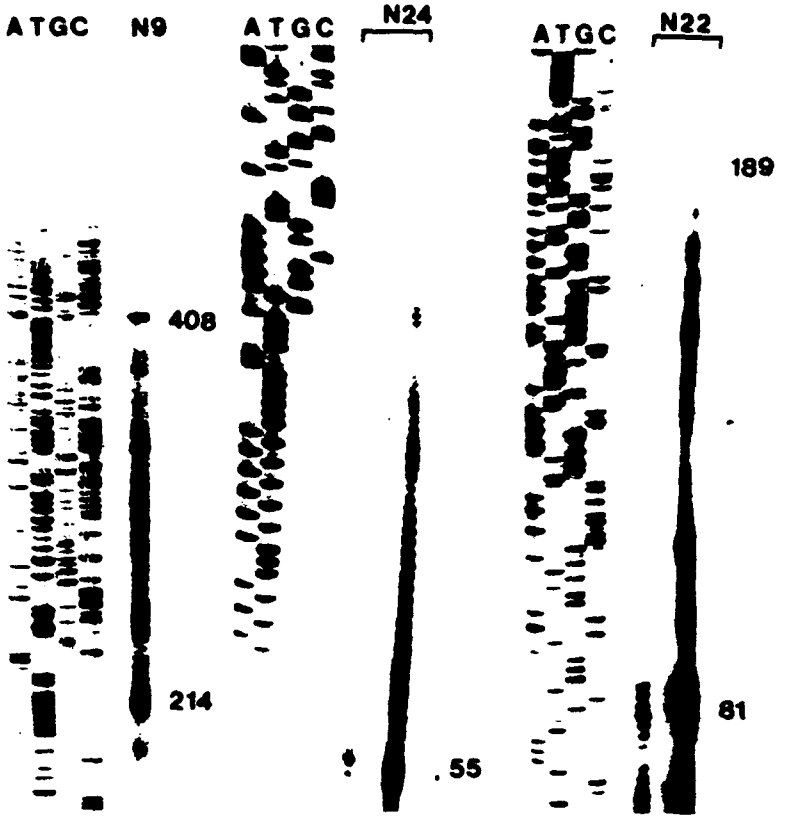


FIG. 5. Location of the transcriptional start site(s). Primer extension analysis of U937 poly(A)⁺RNA with the N-9, N-24, and N-22 primers is shown. The location of each primer is shown in Fig. 2. The N-22 and N-24 primer extension products were loaded adjacent to DNA sequence ladders generated with the same primers, respectively. The N-9 primer extension products are shown next to a DNA sequence ladder. The size of each extension product in nucleotides is indicated.

1987). The sequence located at position −1,235 resembles a DNase I hypersensitive site found in the rabbit uteroglobin gene region (Jantzen *et al.*, 1987). The binding site for the EBP20 protein is found within the SV40 core C element (Johnson *et al.*, 1987) and is present at position −800. Finally the consensus binding sequence for a NF-κB-like factor found in the interleukin-2 receptor α-chain (Leung and Nabel, 1988) is present at position −905. This site is particularly interesting in that κB-like sites are associated with cell-surface glycoproteins (Baldwin and Sharp, 1988).

Within the 591-bp region of intron 1 are found 16 sequence motifs that could potentially interact with DNA-binding proteins. Sequence motifs for a progesterone-responsive element in the chicken lysozyme gene (LysHRE1) (Hecht *et al.*, 1988), and a tissue-specific element in the human α_1-antitrypsin (α_1-AT) gene (De Simone *et al.*, 1987) are present at positions −648 and −564, respectively. Two octamer-binding sequences found in a variety of genes, including the SV40 (Zenke *et al.*, 1986) and immunoglobulin (Augereau and Chambon, 1986) enhancers, are present at positions −530 and −255, respectively. Tandem AATAAAT sequences lie near the 5′ end of the intron. This sequence has been reported to be involved in mediating cell-specific and thyroid hormone stimulation of the rat growth hormone gene (GH-CSE2) (Ye *et al.*, 1988). A DNA motif similar to the liver-specific DNA-binding protein EBP20-ADE11 (Costa *et al.*, 1988) is present at position −440. The sequence found at position −408 is identical to the sequence found within the core enhancer for SV40 (Weiher *et al.*, 1983). The Chinese hamster ovary adenine phosphoribosyl-transferase gene (APRT) contains the sequence GCCCCRCC (Park and Taylor, 1988) which is found at position −272. Multiple PEA3 sequence motifs (Martin *et al.*, 1988) are located at positions −107, −75, and −51. The sequence AGATGACT (Garcia *et al.*, 1987) at position −222 shares homology with the consensus sequence (C/G)TGACT(C/A)A that is recognized by the binding protein AP-1 (Lee *et al.*, 1987). Additionally, the sequence GGTGAGCCTGGG at position −281 is nearly identical to a sequence within the TCI and TCII motifs of the SV40 enhancer (Zenke *et al.*, 1986). Both TCI and TCII can bind the AP-2 protein (Mitchell *et al.*, 1987). The AP-1 and AP-2 sites are responsive to phorbol esters (Angel *et al.*, 1987; Chiu *et al.*, 1987) and, therefore, it will be of particular interest to investigate these sequences as the p150,95 α-subunit gene is upregulated by phorbol esters.

Expression of chloramphenicol acetyltransferase directed by the 5′-flanking region

The results described above showed that transcription is initiated at one or more sites within the region −1,200 to −1,371. To determine if a functional promoter is within this region, the 746-bp *Bam* HI-*Pst* I fragment corresponding to the region −1,020 to −1,766 (Figs. 2 and 6) was inserted upstream of the CAT gene in the promoterless and enhancerless plasmid pCAT-Basic. The resulting plas-

TABLE 2. LOCATION AND IDENTIFICATION OF POTENTIAL REGULATORY ELEMENTS

Regulatory element	*5'→3' Sequences*	*Location*
LVaRS (Speck and Baltimore, 1987)	GAACAG	−2,108
IgHC3 (Augereau and Chambon, 1986)	CCACCA	−2,033, −1,729, −1,237, −383
GTI-SV40 (Siao *et al.*, 1987)	GGGTGTGG	−1,925
NFI-E3 (Garcia *et al.*, 1987)	GGCAA	−1,810, −1,148
PEA3RS (Martin *et al.*, 1988)	AGGAAG	−1,793, −107, −75, −51
SP1 (Dynan and Tjian, 1983)	GGCGGG	−1,598, −1,595, −1,501, −138
TATA (Matsui *et al.*, 1980	TAAAAAT	−1,531
TATA (Matsui *et al.*, 1980)	ATATAA	−1,332
uteroglob.1 (Jantzen *et al.*, 1987)	RYYWSGTG	−1,235
Ad2MLP (Garcia *et al.*, 1987)	TATAAA	−1,099, −605
NFKB CS2 (Leung and Nabel, 1988)	RGGGRMTYYC	−905
EP20-SV40 (Johnson *et al.*, 1987)	TTCCACAC	−800
LysHRE1 (Hecht *et al.*, 1988)	CAGAGAACACAGG	−648
α1AT-CS (De Simone *et al.*, 1987)	TGTGGTTT	−564
oct-B2-SV4 (Zenke *et al.*, 1986)	CTTTGCAT	−530
GH-CSE2 (Ye *et al.*, 1988)	AATAAAT	−1,098, −487
EBP20-ADE11 (Costa *et al.*, 1988)	TCCTACCC	−440
SV40.13 (Weiher *et al.*, 1983)	TGGAAAG	−408
SV40.4 (Zenke *et al.*, 1986)	CCCAGGCTCCCC	−281
APRT cons (Park and Taylor, 1988)	GCCCCRCC	−272
OCTA3 (Augereau and Chambon, 1986)	ATTTGCAT	−255
AP1-E3.2 (Garcia *et al.*, 1987)	AGATGACT	−222

Previously identified DNA-binding proteins (regulatory elements) and the specific DNA sequence these proteins recognize are shown. The location of homologous DNA sequences in the 5'-flanking region is indicated. The numbers correspond to the DNA sequence shown in Fig. 2.

mid, pB4P3-CAT, was transfected into U937 cells by electroporation. It has previously been shown that stimulation of U937 with PMA for 48 hr leads to a marked increase in p150,95 α-subunit mRNA and surface expression (Miller *et al.*, 1986). Therefore, 24 hr after transfection, PMA was added and the cells were assayed for CAT activity 48 hr later. Little or no CAT activity was detected even in the presence of PMA (Fig. 6), indicating that additional flanking DNA was required. To determine if this conclusion was correct, pB 4.1-CAT, which contains approximately 3.6 kb of additional contiguous upstream flanking DNA, was similarly assayed. Stimulation with PMA resulted in an approximately threefold increase of CAT activity over the background levels observed in the absence of PMA

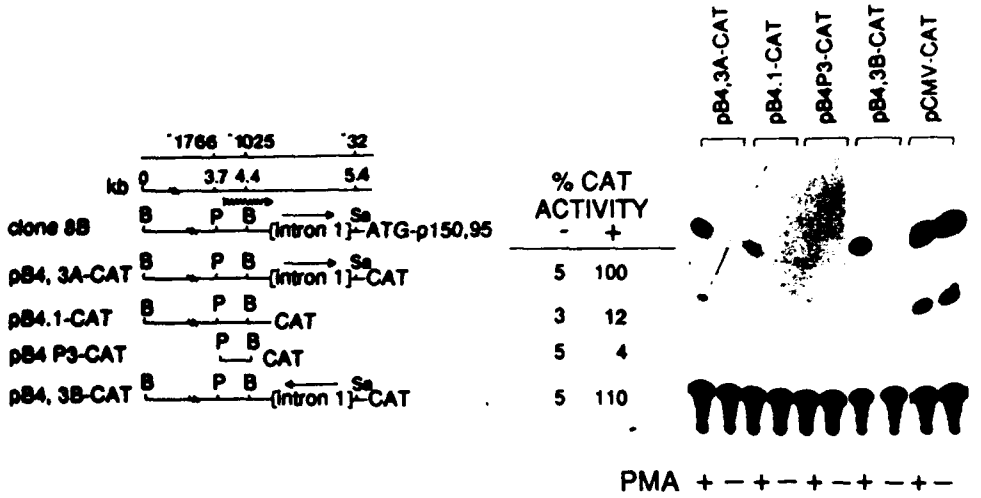


FIG. 6. Functional characterization of the 5'-flanking region. A partial restriction map of the 5'-flanking regioin of the p150,95 α-subunit gene (clone 8B) is shown: B, *Bam* HI; P, *Pst* I; Sa, *Sac* I. The size and location of the 5'-flanking DNA fragments subcloned into pCAT-Basic are shown (not drawn to scale). The region of the 5'-flanking DNA fused to the CAT gene in each construct is indicated. The orientation of intron 1 relative to that in clone 8B is indicated with an arrow. The region of transcription initiation is indicated by a wavy arrow. The autoradiogram shows the results of chromatographic separation of acetylated products formed with 25 μg of cell extract per reaction. The presence (+) or absence (−) of PMA (2 ng/ml) during the transfection is indicated. CAT activity in cells transfected with pB4,3A-CAT in the presence of PMA was defined as 100%. The efficiency of electroporation was monitored with pCMV-CAT as control. The transfections were performed three to five times, and representative data are presented.

(Fig. 6). The identification of a number of DNA sequence motifs for DNA-binding proteins within intron 1 suggested that additional transcriptional control sequences may contribute to the expression of the p150,95 α-subunit gene. The effect of intron 1 on CAT activity was determined after transfection of pB4,3A-CAT into U937. PMA stimulation leads to a 20-fold increase in CAT activity over background levels in the absence of PMA and a 8.5-fold increase over levels detected in the absence of intron 1 (compare pB4.1-CAT with pB4,3A-CAT, Fig. 6). This enhanced stimulation of CAT activity was independent of the orientation of intron 1. CAT activity in U937 cells transfected with either pB4,3A-CAT or pB4,3B-CAT, which contains intron 1 in the opposite orientation, was the same (Fig. 6). The stimulation of CAT activity by PMA was specific to these CAT fusions as the cytomegalovirus enhancer/promoter DNA in the control plasmid pCMV-CAT was shown to efficiently drive CAT gene expression in the absence of PMA.

Determination of the transcriptional start site(s) from the transfected CAT constructs

S1 nuclease analysis was performed on poly(A)⁺RNA isolated from PMA-stimulated U937 cells transfected with either pB4,3A CAT or pB4,3B CAT to determine if transcription was correctly initiated from these constructs. The 5' end-labeled single-stranded probes spanned the 746-bp *Bam* HI-*Pst* I fragment containing the transcriptional initiation site(s) of the endogenous p150,95 γ-subunit gene. The first 12 nucleotides at the 5' end of probe 758 and the first 14 nucleotides at the 5' end of probe 760 (Fig. 7B) are, however, not homologous to the endogenous gene but are instead unique to the CAT constructs. During the construction of pB4,3A CAT and pB4,3B CAT an additional 18 and 41 nucleotides, respectively, were introduced adjacent to the *Bam* HI site at −1,025 in the 5'-flanking region. The 5' end-labeled protected fragments, therefore, correspond to RNA initiated from the CAT constructs only. Three major protected fragments were observed in RNA from cells transfected with pB4,3A CAT. One major fragment was observed in RNA from cells transfected with pB4,3B CAT (Fig. 7A,B). The location of these transcription initiation sites is just downstream of the TATA box at −1,331.

PCR analysis of poly(A)⁺RNA isolated from transfected cells was also done to correeborate the S1 nuclease analysis results. A series of PCR primers was designed successively further upstream of the transcriptional initiation sites of the endogenous p150,95 γ-subunit gene and used in combination with an antisense primer to the CAT gene (Fig. 7C). Sense primers upstream of −1,295 did not yield a PCR product. Sense primers at −1,295 and further downstream, however, yielded PCR products of the expected sizes. This indicates that transcription from these constructs initiates at or near −1,295. S1 nuclease and PCR analysis, taken together, strongly suggest that transcription initiates at or very near the initiation site(s) of the endogenous gene.

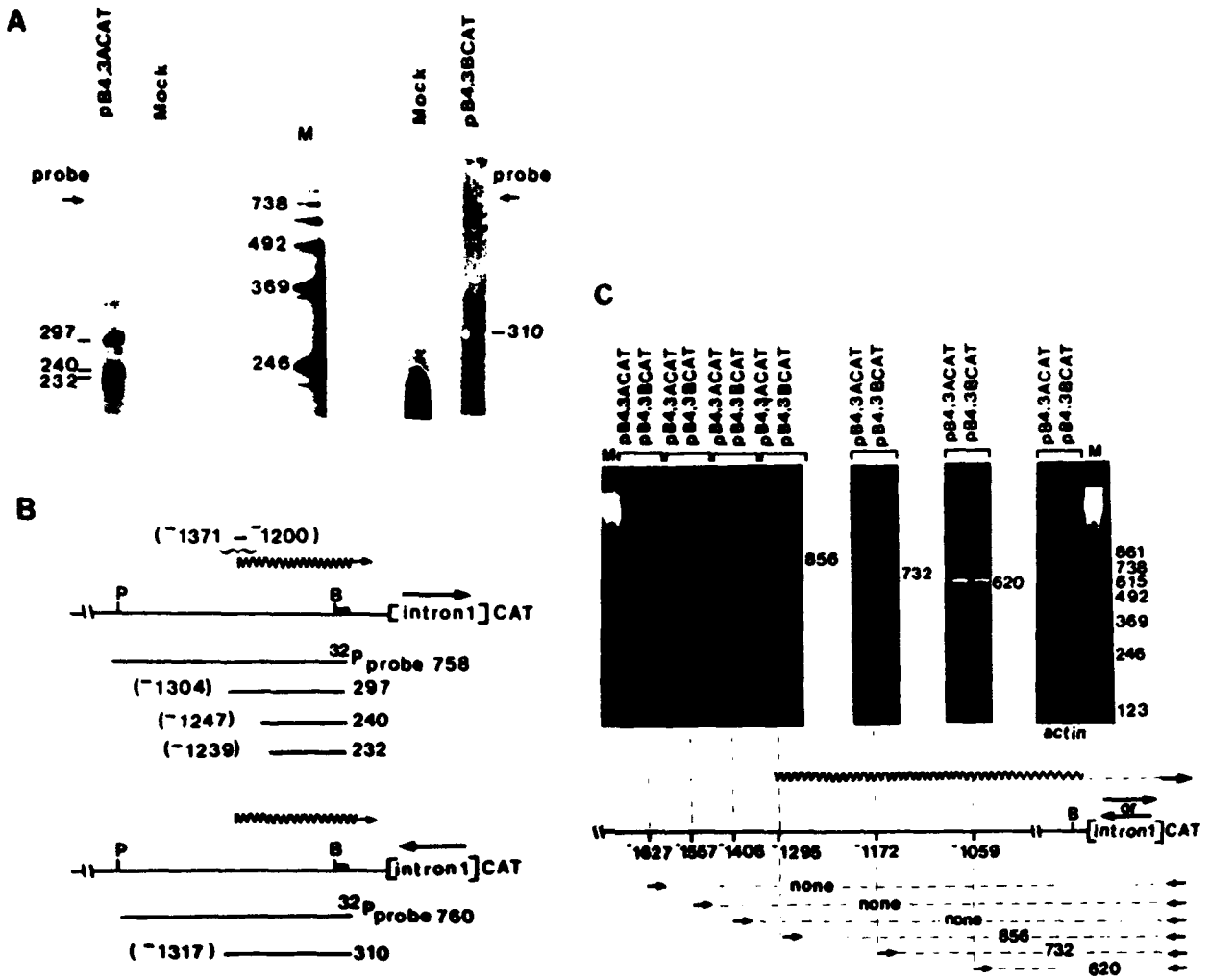


FIG. 7. S1 analysis and PCR analysis to determine transcriptional start sites from transfected CAT constructs. A. Poly(A)⁺RNA was isolated from U937 cells transfected with pB4,3ACAT, pB4,3BCAT, or no DNA (mock) and hybridized to a specific 5′ end-labeled probe. The location of a short stretch of nucleotides in each construct not present in the corresponding region of the endogenous gene is indicated by a black box. Probes 758 and 760 have incorporated this region (see text). After S1 nuclease digestion, the hybrids were analyzed on an 8% polyacrylamide. The major protected products (297, 240, and 232 nucleotides) resulting from transfection with pB4,3ACAT and the major protected product (310 nucleotides) resulting from transfection with pB4,3BCAT are shown. The location of the undigested probes (not visible) is indicated. Lane M, 123-bp ladder. B. Schematics of the S1 nuclease reactions are shown. Probes 758 and 760 span the *Bam* HI (B) and *Pst* I (P) sites at positions −1,025 and −1,766, respectively. The transcriptional start sites determined from the size of each protected fragment are shown in parentheses. The region of transcriptional initiation (−1,371 to −1,200) from the endogenous p150,95 α-subunit gene is shown. Wavy arrow denotes direction of transcription. C. Poly(A)⁺RNA was isolated from U937 cells transfected with pB4,3ACAT or pB4,3BCAT and amplified with a series of PCR primers. The antisense primer was derived from the 5′ end of the CAT gene. The sense primers were derived from the 5′-flanking region of the p150,95 α-subunit gene (−1,627 to −1,059). The size of the PCR product obtained with each primer pair is indicated on the gel photograph and the schematic.

Effect of the 5′-flanking DNA on the SV40 promoter

The presence of an enhancer element within intron 1 was indicated since the stimulation of CAT activity from pB4,3A-CAT and pB4,3B-CAT was independent of the orientation of intron 1. To study the effect of intron 1 on a heterologous promoter, the 1.1-kb *Bam* HI-*Pst* I fragment containing this intron was subcloned approximately 1.6 kb upstream of the SV40 promoter-CAT fusion in the pCAT-promoter plasmid. Since additional sequences upstream of intron 1 were also found to be essential for transcription of the p150,95 α-subunit gene (results of transfection with pB4.1-CAT), it was of interest to determine if the SV40

promoter could also be stimulated by DNA from this region. Therefore, the 4.4-kb *Bam* HI fragment upstream of intron 1 in clone 8B was also subcloned into the pCAT-promoter plasmid. The two resulting constructs, pSV-CAT-1 (containing intron 1) and pSV-CAT-2 (containing the 4.4-kb *Bam* HI fragment) were transfected into HeLa cells. As controls, the pCAT-promoter plasmid and the pCAT-control plasmid (which contains an SV40 enhancer and promoter, Promega, Madison, WI) were also transfected. The pCAT-promoter plasmid was inactive in HeLa cells, however, both pSV-CAT-1 and pSV-CAT-2 expressed high levels of CAT activity (Fig. 8). The stimulation of CAT activity by the 4.4-kb *Bam* HI fragment in pSV-CAT-2 is equivalent to that of the SV40 enhancer in the pCAT-control plasmid. Additionally, the pSV-CAT-2 plasmid expressed 5- to 10-fold more CAT activity than pSV-CAT-1. However, the expression of the CAT gene was not increased by PMA with either construct. These results indicated that one or more enhancers were present within the 5'-flanking DNA of the p150,95 α-subunit gene and that the cellular and promoter specificities of these enhancers are not limited to U937 cells and the p150,95 α-subunit promoter, respectively. Alternatively, the 5'-flanking DNA may encode *trans*-acting factors.

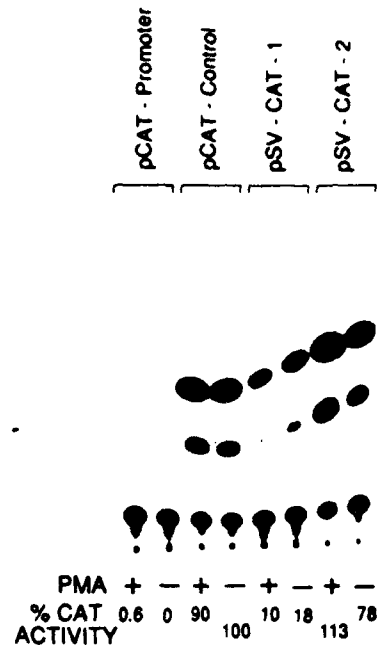


FIG. 8. Effect of the 5'-flanking DNA on the transcriptional activity of the SV40 promoter. HeLa cells were transfected with the indicated plasmid constructs in the presence or absence of PMA (2 ng/ml). Approximately 50 μg of cell extract was assayed per reaction and CAT activity in cells transfected with pCAT-control (which contains both the SV40 promoter and enhancer) in the presence of PMA was defined as 100%.

Analysis of the 3'-flanking sequence

Exon 31 contains the last 102 nucleotides of the translated sequence before the TGA stop codon and extends into the 3' untranslated DNA. To see if other introns are present, the entire untranslated region was sequenced. A comparison of this 1.5-kb sequence with the published 3' untranslated region contained in the cDNA sequence revealed a number of base changes and additions. However, no additional introns were present. The polyadenylation signal sequence, AATAAA, is found at the 3' end (Fig. 9). Within the 178 nucleotides downstream of the AATAAA sequence are three sequences that show homology to previously identified 3' sequence elements. The sequence CACTG is homologous to the sequence CAYTG (Gil and Proudfast, 1987) found 3' of the AATAAA site of many vertebrate genes. The sequence TTGTGAATAT resembles another consensus motif, TTG/ANNNTTTTTT (Birnstiel *et al.*, 1985), which also is present in the region immediately beyond the polyadenylation signal of many genes. Three nucleotides downstream of this second sequence is found a third sequence, TCTCTTGGAA, which is similar to the GT-rich homology TGTGTTGGAA present in the interferon-γ gene (Taya *et al.*, 1982).

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AAAAATAAAAAAGCCTTCTGTGGATATGAGTCCTGAAGGA
TGACACCCATGGGGTCCCTTTACCACGGTGGACCCTGGCC
AGCACTGAGGCCAGGGGGCAGGACAAGAAGTTAACCAGAG
TAGGGTTGTGAATATCCCTCTCTTGGAAGTAACCTGACCT
CTTAATCTGCTCACTCCACTCTCAGGG
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FIG. 9. Nucleotide sequence of the 3'-flanking region. The putative polyadenylation signal is boxed. Sequence motifs present in the 3' regions of other genes are underlined.

DISCUSSION

In this paper we have described the isolation and characterization of a genomic clone for the p150,95 α-subunit gene. Our studies characterizing the exon–intron organization of this gene are in general agreement with a previous report by Corbi *et al.* (1990), with a few exceptions (see below). We provided further characterization of this gene with regard to detailed molecular analysis of the 5' and 3'-flanking regions. In agreement with the above authors, we found that the 31 exons are distributed over approximately 30 kb of chromosomal DNA. The exons are clustered into five groups that correspond to the domains of the p150,95 protein. The signal peptide coding region is split between exon 2 and exon 3. The inserted domain or "I" domain is contained within exons 7, 8, 9, and 10. This domain is speculated to be important in the binding of ligands (Corbi *et al.*, 1987). The integrin receptors require Ca^{2+} or Mg^{2+} to bind ligands. The p150,95 α-subunit se-

quence contains three repeats that are similar to the "EF-hand" loop structure found in other Ca^{2+}-binding proteins. The three metal-binding domains are found in separate exons (exons 14, 15, 16). The transmembrane is split between exons 30 and 31. Exon 30 contains 22 of the 26 amino acids of the transmembrane domain. The cytoplasmic domain is contained in exon 31. However, we found one major difference and a few minor ones over those of Corbi *et al.*. The size of intron 30 in our clone is greater than 1 kb, whereas they reported an intron size of only about 250 bp. Additionally, the sizes of several of the smaller introns (*e.g.*, introns 11, 23, 25, 26, 28) were in general shorter by comparison to those previously reported. Several single base changes were found in the intronic 5′ or 3′ consensus sequences (not indicated); however, all junctions were consistent with the gt-ag rule for splice donor and acceptor sites.

The I domain shares homology to regions found in the von Willebrand factor (vWF) gene (Mancuso *et al.*, 1989), the cartilage matrix protein (CMP) gene (Kiss *et al.*, 1989), the complement factor B gene (Campbell and Porter, 1983), and the type IV collagen α polypeptides, α2 and α3 (Saitta *et al.*, 1990; Stokes *et al.*, 1991). The exon–intron arrangement of the p150,95 α-subunit gene is similar to that of the vWF domain A3 in that four exons of approximately equal sizes encode each of these gene's domains. In contrast, the two repetitive domains found in the CMP gene and the domains in the α2(VI) and α3(VI) genes are each encoded by two exons of dissimilar sizes. As is the case with vWF domain A and the CMP-1 and CMP-2 domains, the introns flanking the I domain are type 1. Exons that have flanking introns of the same phase class are theorized to have arisen by gene segment duplication or exon shuffling. The functional significance of the homology of the I domain with these other domains is unknown. Since the vWF A domain can bind a number of ligands, the I domain conceivably may also interact with similar molecules.

Since the 3′ untranslated sequence was not previously reported, the 1.5-kb DNA sequence immediately following the TGA translational stop codon was completely sequenced. A comparison of the 3′ untranslated sequence of the cDNA sequence (Corbi *et al.*, 1987) was made with the 3′ sequence of our genomic clone. Our genomic clone has an additional 47 nucleotides between the translational stop codon and the putative polyadenylation site was compared to the cDNA sequence; however, they are distributed throughout this region. Therefore, no additional introns are present and the size of exon 31 is at least 1,247 nucleotides.

In further studies the 2,163-bp region preceding the translational start codon was completely sequenced. The presence of an additional intron within the untranslated region was indicated after comparing the cDNA sequence with our genomic sequence. The two sequences diverge 52 nucleotides upstream of the ATG start codon and a 3′ splice sequence is found at this location. However, 65 nucleotides upstream of the ATG codon is found a second 3′ splice sequence that more precisely matches the consensus sequence for a 3′ splice acceptor site. S1 nuclease analysis for this region confirmed the presence of an intron. Moreover, the size of the protected fragments (71 and 88 nucleotides) suggested that both 3′ splice sites might be functional. The abundance of the 88-nucleotide-long fragment was over 10 times greater than the 71-nucleotide-long fragment, which indicates that the second splice site is the preferred site. A 5′ splice sequence (at position −657) and a second putative 5′ splice sequence is present 94 nucleotides further downstream (at position −563). However, primer extension analysis indicated that the 5′ splice sequence at position −657 is the correct site.

Primer extension analysis was used to determine the approximate transcriptional start site. Although there was heterogeneity in the sizes of the extended products, most transcripts were extended to within a 100- to 200-nucleotide region approximately 1,200 nucleotides upstream of the ATG translational start sites. The 5′-flanking sequence was searched for sequences recognized by DNA-binding proteins. Two putative TATA boxes (Matsui *et al.*, 1980) are found within the region assigned to the transcriptional initiation site. Additionally, two putative GC boxes (Dynan and Tjian, 1983) are found flanking the two TATA boxes. It has not been determined from these studies whether the TATA boxes that are present upstream of the most 5′ transcriptional initiation sites are functional. The 746-bp region from −1,020 to −1,766 that contains these sequence motifs could not alone drive transcription of the CAT gene. The inclusion of an additional 3.6 kb of upstream DNA in the pB4.1-CAT construct resulted in a marked increase in expression of CAT activity. Additional 5′ sequences, thus, appear to be essential for expression although the location of these sequences within this large stretch of DNA was not determined. The most dramatic increase in CAT expression resulted by including the region that contains intron 1 in these CAT fusions. The stimulation of CAT activity was independent of the orientation of the intron 1-containing fragment indicating that an enhancer was present. Regulatory elements within the first intron of a gene have been also found in the human 4F2 heavy-chain gene (Karpinsky *et al.*, 1989) and the *Drosophila* β3 tubulin gene (Bruhat *et al.*, 1990). Alternatively, this region may contain a gene(s) for a *trans*-activating factor, as a number of ATG codons were revealed, although the sequence for such a factor would have to be short because a number of translational stop codons are also scattered throughout this region. The orientation-independent enhancement of CAT activity by this region indicates that the promoter for the p150,95 α-subunit gene is not within this region. On the contrary, the orientation of the 4.4-kb *Bam* HI fragment in pB4.1-CAT is critical because the reverse orientation markedly decreases CAT activity thus indicating the presence of a promoter (data not shown). Activation of the SV40 promoter by sequences within the 746-bp *Bam* HI–*Pst* I fragment and the 4.4-kb *Bam* HI fragment further demonstrates the presence of *cis*-acting regulatory regions. Interestingly, although PMA was required for activation by these DNA regions in U937 cells, these sequences were functional in HeLa cells in the absence of PMA.

The 5′-flanking region of the p150,95 α-subunit gene shares some similarities with the regulatory region of the

α4 integrin gene (Rosen *et al.*, 1991). The 5′ untranslated regions of both genes are unusually long—approximately 714–777 nucleotides for p150,95 and 743 nucleotides for α4. The 5′-flanking region of the α4 gene, however, does not contain an intron. ATG codons precede the translational initiation codon in the p150,95 α-subunit gene. Interestingly, translation of the α4 gene begins at the second ATG in exon 1. Such upstream ATG codons are also found in the long 5′ untranslated regions of proto-oncogenes (Kozak, 1987) where they may be involved in translational control. Both AP-1 and AP-2 sites are also found in the α4 gene; however, unlike the arrangement with p150,95 α-subunit gene, they are separated by 961 nucleotides.

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